

## INTRACELLULAR REGULATION OF ENZYME SECRETION FROM RAT OSTEOCLASTS AND EVIDENCE FOR A FUNCTIONAL ROLE IN BONE RESORPTION

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*(Received 8 September 1989)*

### SUMMARY

1. Osteoclasts are known to secrete acid phosphatase, an iron-containing phosphohydrolase. We have investigated (a) the possibility that acid phosphatase has a functional role in bone resorption and (b) the factors controlling enzyme secretion from isolated rat osteoclasts.

2. Osteoclasts were freshly disaggregated from neonatal rat long bones and dispersed at low densities on devitalized cortical bone slices or on plastic substrate. The levels of acid phosphatase in culture medium were measured spectrophotometrically using 4-nitrophenyl phosphate as hydrolysable substrate. The total plan area of bone resorbed was quantified by scanning electron microscopy in combination with image processing and analysis.

3. Ninety-three per cent of the total enzyme activity detected in the supernatant exposed to bone–osteoclast preparations was resistant to inhibition by D-tartaric acid and was bound to an antibody known to be highly specific for the osteoclast-derived isoenzyme, showing that it originated from osteoclasts.

4. A diminution in the level of supernatant enzyme activity achieved by incubating bone–osteoclast preparations with an antiserum specifically binding the osteoclast isoenzyme, or with a non-competitive inhibitor, molybdate or with competitive inhibitors, disphosphonates, led to a marked reduction of osteoclastic bone resorption.

5. The rate of the enzyme released into the culture supernatant, whether from resorbing (cultured on bone) or non-resorbing (cultured on plastic) osteoclasts declined gradually over 22 h, but that from the former was significantly depressed within the first 30 min of incubation. The supernatant enzyme concentration increased linearly up to 3 h; the levels released from resorbing osteoclasts remained consistently lower than those from non-resorbing cells.

6. Exposure of osteoclasts for 18 h to elevated  $[Ca^{2+}]_0$  levels produced a concentration-dependent inhibition of supernatant acid phosphatase levels. In the presence of 20 mM  $[Ca^{2+}]_0$  enzyme secretion from resorbing osteoclasts was significantly lower than that from non-resorbing cells.

7. Exposure of bone-osteoclast preparations to pertussis toxin produced no significant change of acid phosphatase release, while cholera toxin, dibutyryl cyclic AMP and forskolin produced a marked elevation of enzyme secretion. Ionomycin was found to inhibit enzyme release and this was less marked when osteoclasts were incubated on plastic substrate.

8. There was a significant positive correlation between basal enzyme release and bone resorption (correlation coefficient 0.78;  $P < 0.01$ ) and between the percentage fall in supernatant enzyme activity and the percentage reduction of measured resorption in the presence of elevated  $[Ca^{2+}]_o$  (20 mM), ionomycin (10  $\mu$ M) or the immunological and chemical inhibitors of acid phosphatase activity.

9. Three major conclusions follow: (a) the results from both immunological and chemical inhibition of acid phosphatase activity and osteoclastic bone resorption point towards an essential role of the enzyme in the resorptive process, possibly by the removal of pyrophosphate, a natural resorption inhibitor. (b) Enzyme release from the osteoclast appears to be under direct inhibitory control of the  $[Ca^{2+}]_o$  generated as a result of osteoclastic resorption. This suggests the existence of an important self-regulatory mechanism. (c) The intracellular regulation of acid phosphatase release is effected via the intermediacy of G proteins, one of which is cholera toxin sensitive and causes stimulation of enzyme release via a cyclic AMP-dependent pathway.

#### INTRODUCTION

The osteoclast is a cell unique in its capacity to resorb bone (Chambers & Horton, 1984; Chambers, Revell, Fuller & Athanasou, 1984). During resorption the surface of the osteoclast adjacent to the bone shows complex plasmalemmal infoldings (the 'ruffled border') surrounded by a circumferential 'clear zone' where the plasma membrane is closely apposed to the bone substrate (Vaes, 1968; Holtrop & King, 1977). It is assumed that the 'clear zone' represents a region of annular sealing, defining a central micro-compartment between bone and osteoclast that is maintained at a low pH, and in which resorption is effected by the secretion of acid hydrolases and probably free radicals (Vaes, 1968; Lucht, 1971; Doty & Schofield, 1972; Baron, Neff, Louvard & Courtoy, 1985).

One of the acid-optimum hydrolases found in osteoclasts is acid phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1.3.2), a member of a widely distributed class of iron-containing isoenzymes with phosphohydrolase activity. The structures of these isoenzymes have been extensively conserved during evolution (Ketcham, Baumbach, Bazer & Roberts, 1985). An association between the bone-derived isoenzyme of acid phosphatase and bone resorption has been suspected from early observations demonstrating increased levels of enzyme activity in plasma during physiological and pathological states of increased bone remodelling (Campbell & Moss, 1961; Chen, Yam, Janckila, Li & Lam, 1979). This isoenzyme is not inhibited by D-tartaric acid, a competitive inhibitor of prostatic and lysosomal acid phosphatases (Abul-Fadl & King, 1949), and apart from the osteoclast (Minkin, 1982), the enzyme is also expressed normally in differentiated cells of the monohistiocytic series, including alveolar macrophages and Kupffer cells

(Efstratiadis & Moss, 1985*a, b*). The enzyme is not found in significant amounts in precursor monocytes (Efstratiadis & Moss, 1985*b*).

Despite the suspected role of acid phosphatase secretion in bone resorption, it has been difficult to demonstrate that osteoclasts secrete the enzyme: acid phosphatase has been localized histochemically to the 'ruffled border' of the osteoclast, an area where membrane-associated activities required for bone resorption appear to be concentrated. However, evidence has been conflicting as to whether the hydrolase is also present in the extracellular compartment (Lucht, 1971; Doty & Schofield, 1972; Holtrop & King, 1977; Miller, 1985). Bone in organ culture releases acid phosphatase (Vaes, 1965), but this may be due to mechanisms other than secretion, or secretion from cells other than osteoclasts. The major impediment to the investigation of such a fundamental function of osteoclasts has been the absence of an experimental system, whereby osteoclasts can be isolated from bone and incubated in cell culture. Only recently Chambers, Fuller & Darby (1987) have developed simple techniques, which make it possible to investigate the release of tartrate-resistant acid phosphatase from osteoclasts dispersed on glass cover-slips. They have demonstrated that calcitonin is a powerful inhibitor of acid phosphatase secretion (Chambers *et al.* 1987). Using a similar approach, but in addition with disaggregated osteoclasts dispersed on devitalized cortical bone substrate (Chambers *et al.* 1984; Zaidi, Chambers, Bevis, Beacham, Gaines Das & MacIntyre, 1988), we have recently provided preliminary evidence suggesting that the enzyme may play an essential role in bone resorption (Zaidi, Moonga, Moss & MacIntyre, 1989*a*). In the present study we have documented the release of tartrate-resistant acid phosphatase from populations of resorbing and non-resorbing osteoclasts, and have provided further immunological and chemical evidence for an essential function of the enzyme in the resorption of bone. In addition, having recently identified an important regulatory effect of ionized calcium on bone resorption (Datta, MacIntyre & Zaidi, 1989; Zaidi, Datta, Patchell, Moonga & MacIntyre, 1989*b*), we have attempted to examine the possible inhibitory effects of elevated extracellular calcium concentrations on enzyme release. Finally, the intracellular mechanisms involved in the control of acid phosphatase release by G proteins, intracellular calcium and cyclic AMP have also been investigated.

## METHODS

### *Bone-osteoclast cultures*

Human bone obtained from patients who died without evidence of bone disease was cleaned and the cortex cut longitudinally with a low-speed saw (Buehler, IL, USA). The pieces of bone were then cut into slices (3 mm<sup>2</sup>), sonicated in sterile distilled water and stored dry at room temperature. Newborn Wistar rats were killed by cervical dislocation and the tibiae and femora were removed and adherent soft tissue was cleaned off. Osteoclasts were mechanically disaggregated by curetting the long bones into HEPES-buffered medium 199 containing heat-inactivated fetal calf serum (FCS; 10%, v/v; GIBCO, UK), glutamine, penicillin (10 i.u. ml<sup>-1</sup>) and streptomycin (100 µg ml<sup>-1</sup>) (all from Flow Laboratories, UK, Ltd). The cell suspension was agitated with a pipette and larger bone fragments allowed to settle for 10 s. The cells were then dropped onto the plastic substrate or slices of devitalized human cortical bone placed in a well of Sterilin (100) 18 mm multiwell dishes. The cells were allowed to sediment and attach to the substrate for 30 min (37 °C). The slices were then washed in minimal essential medium (MEM) containing FCS (10%), glutamine, penicillin, streptomycin (as above) and sodium bicarbonate (0.85 g l<sup>-1</sup>) and placed in separate wells containing

2 ml of the same medium. The cultures were incubated for 18 h (37 °C; 10 % humidified CO<sub>2</sub>) with or without added test substance. Though we incubate our cultures initially at physiological pH (7·4), we do not routinely measure the pH over the 18 h incubation period. Changes in pH in our cultures are likely to be small due to the small number of acid-secreting osteoclasts. After incubation, the culture medium was sampled and the slices were fixed with glutaraldehyde (10 % v/v) and stained with Toluidine Blue. The latter enabled the assessment of multinucleate osteoclasts and mononuclear cells to be identified. The slices were then bleached by immersion in sodium hypochlorite solution (10 %, v/v) and dehydrated in ethanol. In some experiments the bone-osteoclast preparation was critical-point dried and dehydrated through graded ethanol solutions. This method provided a better image of the osteoclast and its excavation. The bone slices were finally sputter-coated with gold and examined under a scanning electron microscope (Cambridge 360, Cambridge Instruments, Bar Hill, Cambs). The total plan area of bone resorbed and the number of osteoclastic excavations was assessed by tracing the outline of each excavation into a digitizer linked to an IBM-AT-controlled image processor (Sight Systems, Newbury, Berks).

#### *Measurement of acid phosphatase activity*

Total acid phosphatase activity was measured by incubating samples (usually 0·15 ml) of medium exposed to osteoclasts, cultured either on bone or plastic, with 4-nitrophenyl phosphate (final concentration 10 mM) in citrate buffer (pH 5·0; 100 mM) at 37 °C for 30 min in a total volume of 0·45 ml. The reaction was stopped and the coloured quinonoid form of the 4-nitrophenate ion was developed by the addition of 0·2 ml sodium hydroxide (0·5 mM) before absorptiometry at 405 nm. Catalytic activity concentrations (micromole substrate hydrolysed per minute (U) per litre sample) were calculated from the absorbance coefficient of 4-nitrophenate,  $\epsilon = 1\cdot85\text{ l mmol}^{-1}\text{ mm}^{-1}$ . In order to measure tartrate-resistant acid phosphatase, D-tartaric acid was added to the incubation medium to achieve a final concentration of 150 mM.

#### *Anti-uteroferrin antiserum*

An antiserum against purified porcine uteroferrin (an iron-containing protein with acid phosphatase activity) had been raised in rabbit and anti-uteroferrin IgG antibodies purified by specific immunoaffinity chromatography as described previously (Echetebe, Cox & Moss, 1987). The antiserum had been shown to cross-react completely with human tartrate-resistant acid phosphatase from Gaucher's disease spleen and alveolar macrophages, and with the increased acid phosphatase activity in sera of patients with osteoclastic bone diseases. The antibody-isoenzyme complex has been shown to possess markedly reduced catalytic activity. The antiserum or immunopurified antibodies also have been found not to cross-react with other acid phosphatase isoenzymes, including the tartrate-resistant erythrocyte enzyme (Echetebe *et al.* 1987). The specificity of the antibody to acid phosphatase has been further confirmed by electrophoresis of an unfractionated extract of Gaucher's disease spleen on SDS(sodium dodecyl sulphate)-polyacrylamide gel followed by probing with anti-uteroferrin antiserum and <sup>125</sup>I-labelled protein A. This has shown that the antiserum reacts only with the 37 kDa protein band corresponding to the iron-containing acid phosphatase isoenzyme and with no other protein (Echetebe *et al.* 1987).

The cross-reactivity of anti-porcine uteroferrin antibody with rat osteoclastic acid phosphatase was confirmed in the following way. Osteoclasts were allowed to settle on a polystyrene surface and the supernatant medium was recovered after incubation at 37 °C. Purified anti-uteroferrin antibodies bound to Sepharose 4B particles were then added to the medium. After standing on ice for 2 h, the particles were sedimented in a microfuge. Total and tartrate-resistant acid phosphatase activities were measured in the culture medium and in the supernatant after sedimentation of the particle-bound antibody.

### RESULTS

#### *Acid phosphatase release from isolated rat osteoclasts*

Culture medium exposed to osteoclasts for 3 h on plastic substrate contained 1·45 U l<sup>-1</sup> total acid phosphatase activity, of which 0·815 U l<sup>-1</sup> was resistant to inhibition by D-tartaric acid. Following sedimentation with Sepharose-linked immunopurified anti-uteroferrin antibody, 0·290 U l<sup>-1</sup> activity remained in the supernatant of which 0·020 U l<sup>-1</sup> was tartrate resistant. Thus the antibody removed

0.795 U l<sup>-1</sup> (93 % of the tartrate-resistant activity). The non-bound fraction of total activity recovered from the supernatant was presumed to be due to unreactive lysosome- and erythrocyte-derived acid phosphatases. Estimates of tartrate-labile ('lysosomal') acid phosphatase are useful indicators of cell lysis.

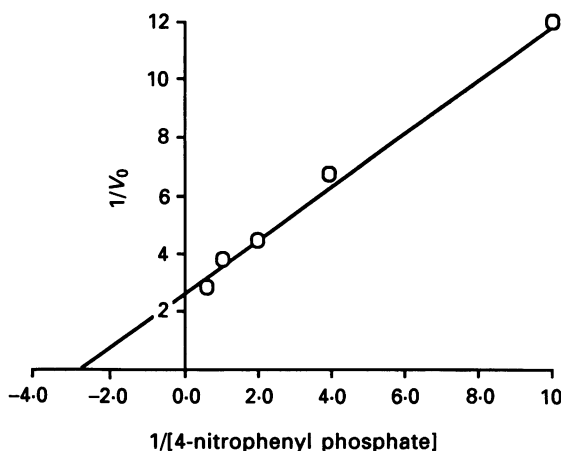


Fig. 1. Lineweaver-Burk plot of data derived from incubation of 150  $\mu$ l cell supernatant (obtained after exposure to bone-osteoclast cultures) with 4-nitrophenyl phosphate (10 mM) in citrate buffer (pH 5.0, 100 mM) in the presence of 150 mM-D(+)tartaric acid for 30 min at 37 °C.

The tartrate-resistant acid phosphatase derived from osteoclast culture supernatants displayed Michaelis-Menten kinetics with a  $K_m$  of 0.35 mM (Fig. 1). This is similar to that found using the same substrate (4-nitrophenyl phosphate) for partially purified tartrate-resistant phosphatases for normal bone and lung (Efstratiadis & Moss, 1985a, b), as well as from Paget's disease serum, osteoclastomas (Hayman, Warburton, Pringle, Coles & Chambers, 1989), Gaucher's disease spleen (Chambers, Peters, Glew, Lee, McCafferty, Mercer & Wenger, 1978) and hairy cells of leukaemic reticuloendotheliosis (Yam, Li & Finkel, 1972).

#### *Effect of immunological and chemical inhibition of acid phosphatase activity on osteoclastic bone resorption*

The tartrate-resistant acid phosphatase activity in the medium was reduced from 2.93 to 1.95 U l<sup>-1</sup> when immunopurified anti-uteroferrin antibodies (60 mg l<sup>-1</sup>) were added to bone-osteoclast cultures. This was accompanied by a marked decrease in the area of bone resorbed and the total number of osteoclastic excavations. There was no effect on the size of the individual excavations (Table 1). A similar effect on resorption was noted when osteoclasts were incubated with whole-rabbit anti-uteroferrin antiserum. Non-immune rabbit serum and a purified antibody against vasoactive intestinal polypeptide, used as controls, failed to inhibit both enzyme activity and bone resorption. The addition of purified spleen acid phosphatase (10 U l<sup>-1</sup>) partially reversed the effect of the specific antibody on bone resorption (Table 1).

Further evidence for the essential role of acid phosphatase in bone resorption was obtained by adding Tris-buffered molybdic acid to the culture medium. Ionic

molybdate is a non-competitive inhibitor of iron-containing acid phosphatases (Lam & Yam, 1977), and its addition to bone-osteoclast preparations caused a concentration-dependent inhibition of the plan area of bone resorbed and the number of osteoclastic excavations; this was accompanied by a reduction of the supernatant acid phosphatase activity by approximately 50% at a concentration of 100  $\mu\text{M}$ .

TABLE 1. The effect of various treatments on the number of osteoclastic excavations per bone slice, mean area of bone resorbed per bone slice, the size of individual excavations per bone slice (expressed as percentage control mean) and the supernatant concentration of acid phosphatase (total, tartrate resistant and tartrate labile)

	Number per slice	Area per slice	Size	Supernatant acid phosphatase (U l <sup>-1</sup> )		
Treatment	(Percentage of control)			Total	Tartrate resistant	Tartrate labile
Expt 1						
Control	100 ± 14.7	100 ± 21.0	100 ± 17.8	3.71 ± 0.2	2.99 ± 0.25	0.72 ± 0.09
Ab (60 mg l <sup>-1</sup> )	48 ± 12.3*	38.0 ± 8.93**	96 ± 14.4	2.62 ± 0.16*	2.01 ± 0.11*	0.61 ± 0.07
AcP (10 U l <sup>-1</sup> )	60 ± 15.5*	68.0 ± 18.9**	102 ± 16.3	—	—	—
AcP + Ab	63 ± 17.0*	71.0 ± 6.73*	94 ± 12.6	—	—	—
Expt 2						
Control	100 ± 12.7	100 ± 18.5	100 ± 13.9	5.07 ± 1.0	4.59 ± 0.56	0.48 ± 0.12
Mo (1 μM)	37.5 ± 19**	110 ± 10.6	110 ± 18.4	3.97 ± 0.9*	3.02 ± 0.62*	0.95 ± 0.23*
Mo (10 μM)	7.1 ± 3.4**	28.0 ± 6.91**	98 ± 12.4	3.88 ± 0.46*	3.22 ± 0.30*	0.66 ± 0.38
Mo (100 μM)	3.5 ± 1.2**	5.0 ± 3.66**	94.6 ± 4.5	2.91 ± 0.5**	2.16 ± 0.30**	0.75 ± 0.15
Expt 3						
Control	100 ± 12.75	100 ± 19.2	100 ± 23.3	10.26 ± 1.13	9.91 ± 2.12	0.35 ± 0.09
EHDP (1 μM)	28 ± 11.2	88.9 ± 30.0	106 ± 18.0	6.55 ± 0.93*	6.01 ± 1.21*	0.54 ± 0.1
EHDP (10 μM)	21 ± 8.9	66.9 ± 28.5*	123 ± 17.9	4.66 ± 0.61**	4.14 ± 0.45**	0.52 ± 0.08
APD (1 μM)	35 ± 14.5	50 ± 23.5**	95 ± 16.4	6.91 ± 1.72*	6.89 ± 2.31**	0.02 ± 0.03**
APD (10 μM)	5 ± 3.5	11.1 ± 4.3**	93 ± 30.2	5.75 ± 1.15**	5.01 ± 0.22*	0.70 ± 0.19

Bone slices per variable in experiments 1, 2 and 3: 5, 6 and 6, respectively. Osteoclasts per slice: control; 5.7  $\pm$  1.0; Ab, 3.6  $\pm$  1.0; 100  $\mu\text{M}$ -Mo, 5.25  $\pm$  0.77; 10  $\mu\text{M}$ -EHDP, 4.9  $\pm$  0.6; 10  $\mu\text{M}$ -APD, 5.9  $\pm$  0.58. There was no morphological evidence of cell toxicity. Abbreviations as follows: Ab, immunopurified anti-uteroferrin antibody; AcP, spleen acid phosphatase; Mo, molybdic acid; EHDP, ethane-1-hydroxy-1,1-diphosphonate; APD, 3-amino-1-hydroxypropylidene-1,1-diphosphonate. All values represent means  $\pm$  S.E.M.

Statistics determined using Student's *t* test: \**P* < 0.05; \*\**P* < 0.01.

molybdate ions (Table 1). In these experiments molybdate was added to the enzyme assay mixture to maintain the concentration of this reversible inhibitor at the same level as in the culture medium. There was a significant correlation between the percentage reduction of bone resorbed and the percentage reduction of supernatant enzyme activity using the antibody and molybdate. The antiserum, immunopurified antibody or molybdate ions did not appear to affect osteoclast number or viability (Table 1) since the number of osteoclasts per bone slice on the molybdate- or antibody-treated slices compared with the respective controls remained unchanged, while in addition, the lysosomal isoenzyme concentration in the medium remained unchanged in treated compared to control (untreated) samples (Table 1). There was a slight increase (*P* < 0.05) of lysosomal acid phosphatase with 1  $\mu\text{M}$ -molybdate ions, but higher concentrations of the cation failed to affect enzyme levels. The results

with immunological and chemical inhibition of bone resorption and acid phosphatase activity confirm and extend those of Zaidi *et al.* (1989*a, b*). The data from both studies have been combined in calculating the correlation coefficients given in Fig. 3*C*.

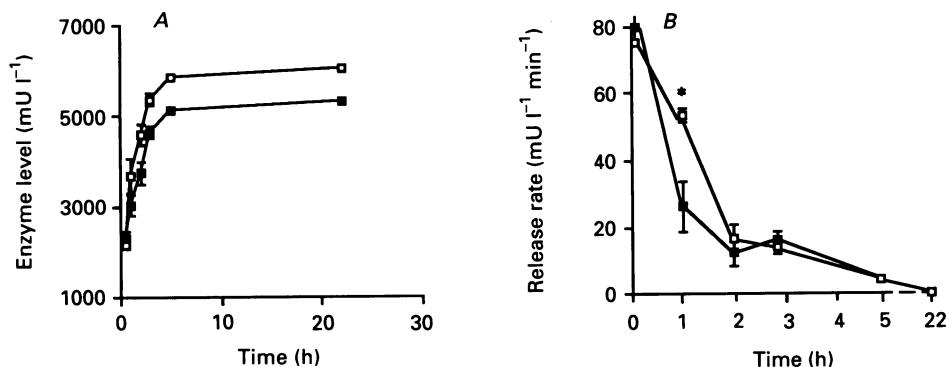


Fig. 2. Time course of the accumulation of tartrate-resistant acid phosphatase in the supernatant (A; mU l<sup>-1</sup>) and the rate of enzyme release (B; mU l<sup>-1</sup> min<sup>-1</sup>) from osteoclasts settled on bone (■) or plastic (□). \*  $P < 0.01$ .

In order to competitively inhibit acid phosphatase action we used two disphosphonates, characterized by the presence of a P-C-P bond: 3-amino-1-hydroxypropylidene-1,1-disphosphonate (ADP) and ethane-1-hydroxy-1,1-disphosphonate (EHDP) in similar experiments. Both compounds reduced bone resorption (plan area of resorption per slice and number of excavations) and the supernatant enzyme concentration in a concentration-dependent manner (Table 1).

#### *Time course of acid phosphatase release and the effect of substrate*

Acid phosphatase released over 22 h was assessed by sampling the culture supernatant from wells of a Sterilin multiwell dish in which osteoclasts were incubated either on bone or on plastic. The samples were obtained 0.5, 1, 2, 3, 5 and 22 h following incubation from four replicate wells; each well contained either five 20 mm<sup>2</sup> bone slices or five 25 mm<sup>2</sup> plastic cover-slips. After 22 h incubation, the osteoclasts looked viable and healthy, but the numbers of osteoclasts per bone slice were reduced by some 20%. The level of tartrate-labile lysosomal acid phosphatase was not significantly different from that seen at 30 min. The cumulative enzyme concentration in the supernatant increased linearly over the first 3 h and was followed by a small but significant elevation ( $P < 0.01$ ; *t* test) over the next 2 h, with minimal accumulation over the next 17 h of incubation (Fig. 2A). This was consistent with a gradual decline in the rate of acid phosphatase secretion over the 22 h period (Fig. 2B). It is notable that the supernatant enzyme concentration of the bone-osteoclast cultures remained significantly lower than that of osteoclast cultures on plastic (Fig. 2A). This was because of the marked reduction, observed within the first hour of incubation, in the rate of enzyme secretion from resorbing osteoclasts (on bone) compared to non-resorbing osteoclasts (on plastic) (Fig. 2B). The cumulative

tartrate-resistant enzyme activity released during the 22 h incubation period correlated significantly with total area of bone resorbed ( $r = 0.78$ ,  $P < 0.01$ ) (Fig. 3A).

*Regulation of acid phosphatase release by extracellular ionized calcium*

The exposure of bone-osteoclast cultures to elevated extracellular calcium levels caused a concentration-dependent diminution both of the supernatant enzyme

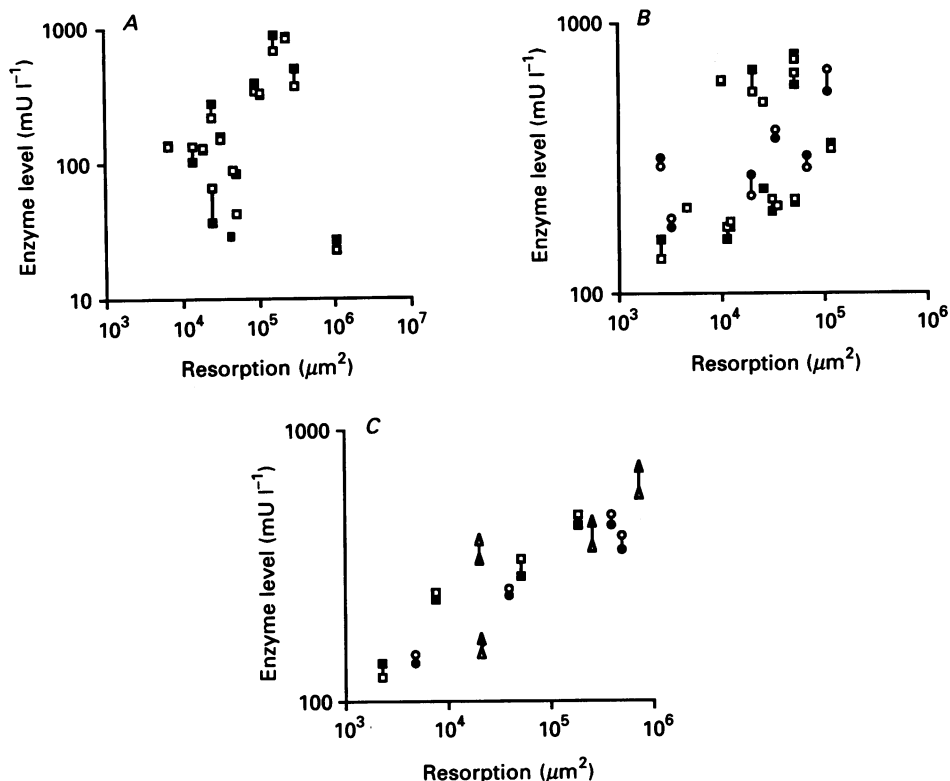


Fig. 3. Correlation between resorption on five or six bone slices and amount of acid phosphatase released by untreated resorbing osteoclasts (A;  $r = 0.78$ ;  $P < 0.01$ ), osteoclasts exposed to elevated extracellular calcium concentrations ( $\circ$ ,  $\bullet$ ;  $r = 0.71$ ;  $P < 0.01$ ) or ionomycin ( $\square$ ,  $\blacksquare$ ;  $r = 0.8$ ,  $P < 0.01$ ) (B) or osteoclasts incubated with immunopurified anti-uteroferrin antibodies ( $\square$ ,  $\blacksquare$ ), molybdate ions ( $\circ$ ,  $\bullet$ ) or diphosphonates ( $\triangle$ ,  $\blacktriangle$ ) (C,  $r = 0.9$ ,  $P < 0.01$ ). The closed and open symbols refer to the total and tartrate-resistant acid phosphatase levels, and their difference relates to lysosomal acid phosphatase levels.

concentration and the plan area of bone resorption, without any effect on cell viability or number (Table 2). There was a significant statistical regression of the measured response on the concentration of ionized calcium ( $P < 0.01$ ; obtained using logistic regression analysis, by comparing responses at each dose to control responses). The percentage fall in the supernatant enzyme concentration was found to correlate significantly with the percentage reduction of measured resorption ( $r = 0.71$ ;  $P < 0.01$ ) (Fig. 3B).

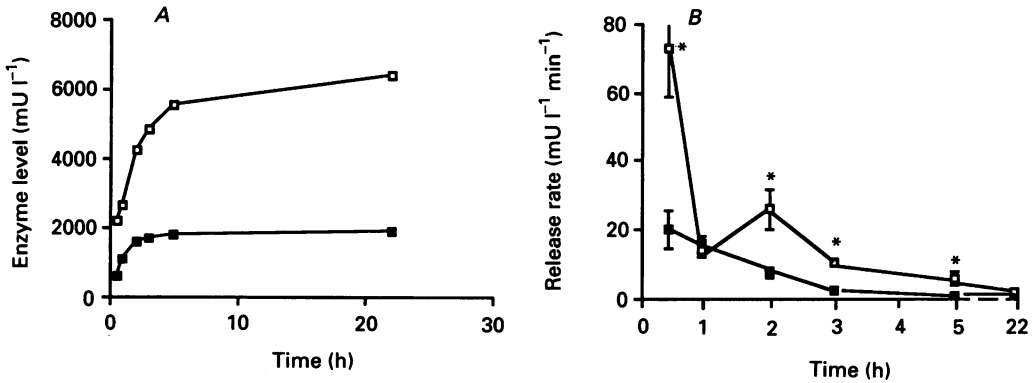


Fig. 4. Time course of the effect of elevation of extracellular calcium on the accumulation of tartrate-resistant acid phosphatase in the supernatant (A; mU l<sup>-1</sup>) and on the rate of enzyme release (B; mU l<sup>-1</sup> min<sup>-1</sup>) from osteoclasts settled on bone (■) or plastic (□). \**P* < 0.01.

TABLE 2. The effect of extracellular calcium elevation or the addition of ionomycin on the number of osteoclastic excavations per bone slice, mean area of bone resorbed per bone slice and the size of individual excavations per bone slice (expressed as percentage control mean), mean osteoclast number per bone slice and the supernatant concentration of acid phosphatase (total and tartrate resistant; expressed as test:control ratio)

Treatment	Number per slice	Area per slice	Size	Supernatant acid phosphatase (U l <sup>-1</sup> )		
				(Percentage of control)		
				Cell number per slice	Total	Tartrate resistant
<b>Expt 1</b>						
Control	100 ± 25.2	100 ± 37.8	100 ± 23.6	5.8 ± 1.0	1.0 ± 0.308	1.0 ± 0.240
Ca <sup>2+</sup> (4 mM)	11.3 ± 9**	19.7 ± 9.2**	108 ± 29.3	6.0 ± 0.86	0.87 ± 0.15	0.97 ± 0.22
Ca <sup>2+</sup> (10 mM)	13.5 ± 10**	12.5 ± 11**	106 ± 10.5	5.2 ± 1.2	0.72 ± 0.22*	0.67 ± 0.09*
Ca <sup>2+</sup> (20 mM)	3.6 ± 2**	1.9 ± 1.3**	109 ± 21	5.4 ± 1.8	0.68 ± 0.23*	0.66 ± 0.25*
<b>Expt 2</b>						
Control	100 ± 27.3	100 ± 33.8	111 ± 15.2	4.6 ± 1.1	1.0 ± 0.27	1.00 ± 0.27
Ionomycin (1 μM)	58 ± 10.8*	44.3 ± 9.8**	96 ± 13.7	5.1 ± 1.2	1.015 ± 0.32	1.040 ± 0.26
Ionomycin (10 μM)	47 ± 13**	34.1 ± 10.5**	104 ± 13.6	4.4 ± 0.9	0.806 ± 0.24*	0.72 ± 0.23*
Ionomycin (100 μM)	0	0	—	3.1 ± 1.7**	2.403 ± 0.60*	1.989 ± 0.5*

Number of bone slices in experiments 1 and 2: 8 and 20, respectively (results from five experiments have been pooled). There is a significant elevation of lysosomal acid phosphatase levels (total—tartrate resistant) between control cultures ( $0.25 \pm 0.021$  U l<sup>-1</sup>, mean supernatant concentration ± s.e.m.) and those treated with 100 μM-ionomycin ( $0.54 \pm 0.045$  U l<sup>-1</sup>), suggesting cell toxicity. Values represent means ± s.e.m.

Statistics determined using Student's *t* test: \**P* < 0.05, \*\**P* < 0.01.

The response to 20 mM-extracellular calcium concentrations over 22 h was assessed, as described above, by sampling at timed intervals culture supernatant from wells of Sterilin (100) multiwell dish in which osteoclasts were incubated either on bone or plastic substrates. We found that in comparison with non-resorbing cells

(on plastic) exposed to 20 mM-ionized calcium or resorbing cells (on bone) exposed to physiological calcium concentrations (1 mM) (see Figs 2*A* and 4*A*), the exposure of resorbing osteoclasts to 20 mM-ionized calcium extracellularly was followed by a marked threefold reduction of the supernatant enzyme concentration; the enzyme

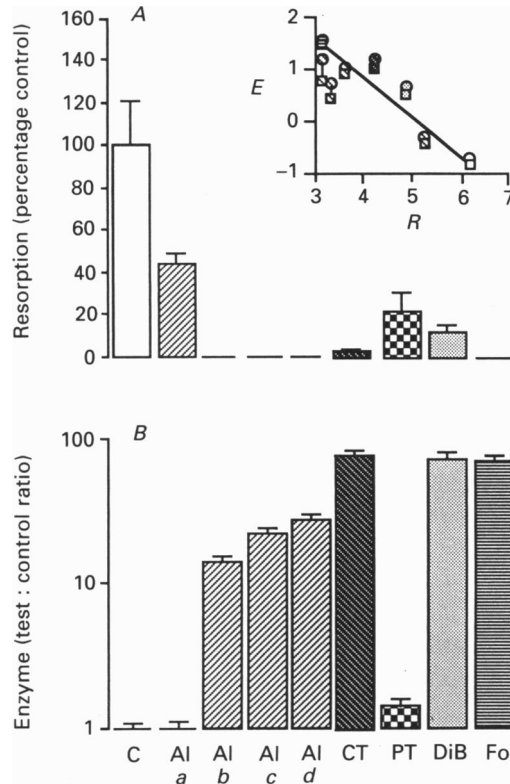


Fig. 5. The effect of aluminium fluoride ions (Al, concentrations as below), cholera toxin (CT,  $5 \mu\text{g ml}^{-1}$ ), pertussis toxin (PT,  $1 \mu\text{g ml}^{-1}$ ), dibutyryl cyclic AMP (DiB,  $100 \mu\text{M}$ ) and forskolin (Fo,  $10 \mu\text{M}$ ) on the total area of bone resorbed per slice (*A*; expressed as a percentage of control means  $\pm$  S.E.M.,  $n = 6$ ) and supernatant tartrate-resistant acid phosphatase concentration (*B*; expressed as test:control ratio  $\pm$  S.E.M.,  $n = 6$ ) after 22 h incubation of the bone-osteoclast cultures. The inset shows the negative correlation between bone resorption (*R*, log of area resorbed per slice) and supernatant enzyme concentration (*E*, log concentration). The squares and circles in the inset refer to the total and tartrate-resistant acid phosphatase levels, and their difference relates to lysosomal acid phosphatase levels. Concentrations: *a*, 1 mM-NaF + 1 mM- $\text{AlCl}_3$ ; *b*, 5 mM-NaF +  $5 \mu\text{M}$ - $\text{AlCl}_3$ ; *c*, 10 mM-NaF +  $10 \mu\text{M}$ - $\text{AlCl}_3$ ; *d*, 50 mM-NaF +  $50 \mu\text{M}$ - $\text{AlCl}_3$ . C represents control.

concentration remained low over the 22 h sampling period (Fig. 4*A*). This was accompanied within the first half-hour by a marked reduction in the rate of enzyme released into the culture supernatant (Fig. 4*B*). It is notable that both the rate of enzyme release and the cumulative concentration were similar in instances either when osteoclasts were incubated on bone at physiological calcium concentrations (1 mM) or when cultured on plastic in the presence of 20 mM-calcium.

*Regulation of acid phosphatase release by G proteins and intracellular second messengers*

The exposure of bone-osteoclast preparations for 22 h to aluminium fluoride anions ( $\text{AlF}_4^-$ ) led to a marked concentration-dependent elevation of acid

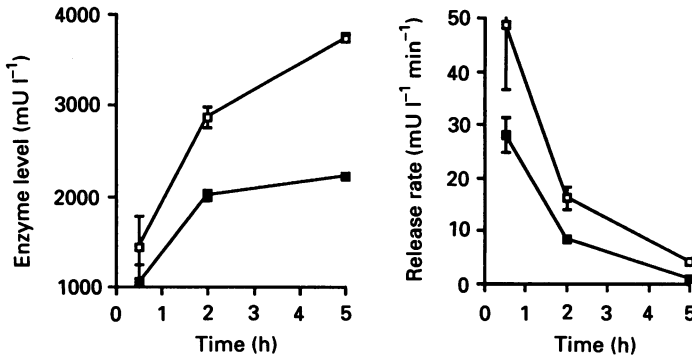


Fig. 6. Time course of the effect of ionomycin ( $1 \mu\text{M}$ ) on the accumulation of tartrate-resistant acid phosphatase in the supernatant (A;  $\text{mU l}^{-1}$ ) and on the rate of enzyme release (B;  $\text{mU l}^{-1} \text{min}^{-1}$ ) from osteoclasts settled on bone (■) or plastic (□).

phosphatase activity in the culture supernatant accompanied by a dramatic reduction of the plan area of osteoclastic resorption. Whereas both cholera toxin ( $5 \mu\text{g ml}^{-1}$ ) and pertussis toxin ( $1 \mu\text{g ml}^{-1}$ ) caused a marked depression of bone resorption, pertussis toxin had no significant effect on enzyme release, while cholera toxin caused an almost 100-fold elevation of supernatant acid phosphatase activity. Similar paradoxical effects on bone resorption and enzyme release were noted following 22 h incubation of bone-osteoclast preparations with dibutyryl cyclic AMP (a diesterase-resistant analogue of cyclic AMP) ( $100 \mu\text{M}$ ) or forskolin ( $10 \mu\text{M}$ ): there was a marked 90% reduction of osteoclastic bone resorption and a 90-fold stimulation of enzyme release. There was a significant inverse correlation between the change in bone resorption and the change of enzyme release (Fig. 5).  $\text{AlF}_4^-$  ions, pertussis and cholera toxins, dibutyryl cyclic AMP and forskolin had no effect on cell viability, osteoclast number per slice or 'lysosomal' acid phosphatase levels, compared with untreated cultures.

The elevation of cytosolic free calcium levels by incubating bone-osteoclast cultures with ionomycin caused a concentration-dependent inhibition of osteoclastic bone resorption and reduction of the supernatant enzyme concentration. However, at  $100 \mu\text{M}$ -ionomycin there was a marked reduction of osteoclast number and a marked elevation of 'lysosomal' acid phosphatase, indicating cell lysis (Table 2). The incubation of osteoclasts on bone or plastic for 5 h in the presence of  $10 \mu\text{M}$ -ionomycin resulted in a marked inhibition of supernatant enzyme concentration. In decreasing order the rate of enzyme released was as follows: ionomycin-treated osteoclasts on bone > ionomycin-treated osteoclasts on plastic > untreated osteoclasts on bone > untreated osteoclasts on plastic (Fig. 6A and B). There was a

marked correlation between the enzyme released and bone resorption ( $r = 0.8$ ,  $P < 0.01$ ).

#### DISCUSSION

##### *On the essential role of 'osteoclastic' acid phosphatase in bone resorption*

A variety of acid hydrolases can be identified histochemically in osteoclasts (Lucht, 1971; Doty & Schofield, 1972; Miller, 1985). During bone resorption, osteoclasts secrete these enzymes in a hormone-responsive manner (Miller, 1985; Chambers *et al.* 1987), and increased concentrations are detectable in serum in physiological and pathological states of increased bone remodelling (Campbell & Moss, 1961; Chen *et al.* 1979). We have demonstrated that isolated rat osteoclasts incubated either on bone ('resorbing') or on plastic ('non-resorbing') release into the medium an acid-optimal phosphohydrolase, acid phosphatase. This enzyme is relatively specific to the osteoclast, is resistant to inhibition by D-tartaric acid and specifically reacts with an antibody raised against a structurally similar protein, uteroferrin. The specificity of the latter antibody to the osteoclastic enzyme has formed the basis of distinguishing, in our culture supernatant, the osteoclast-derived enzyme from other contaminating tartrate-resistant isoenzymes, such as the erythrocyte form. The fraction of the total acid phosphatase activity that is inhibited by tartaric acid is of lysosomal origin (or 'lysosomal' acid phosphatase); this enzyme is as widely distributed as lysosomes themselves (Efstratiadis & Moss, 1985*a, b*).

The role in bone resorption of the majority of osteoclastic acid hydrolases, including that of acid phosphatase, is unknown. The osteoclast is a cell specialized for digestion of a particular substrate of a unique composition, so that it is likely to have developed specialized acid hydrolases that differ significantly from hydrolases from other cells. This appears to be the case for the osteoclastic tartrate-resistant acid phosphatase. We suggest that this enzyme has a primary role in the process of bone resorption by osteoclasts. Our conclusion stems from observations demonstrating a marked reduction of bone resorption due to the inhibition of its activity either by a specific anti-uteroferrin antibody that reduces the number of active enzyme molecules present (Echtebu *et al.* 1987), or by molybdate, a potent inhibitor of the activity of this class of iron-containing phosphatases (Ketcham *et al.* 1985). Both compounds were also found to inhibit markedly supernatant acid phosphatase activity, although the measured concentration of the enzyme might not precisely reflect levels at the cell-bone interface. The plan area of resorption also correlated with supernatant acid phosphatase levels, strengthening our hypothesis for an essential role for acid phosphatase in the resorptive process. We are presently not certain about the exact mechanism of acid phosphatase action in bone resorption, but the evidence provided strongly points towards a catalytic role in the removal of pyrophosphate (P-O-P), a potent natural inhibitor of hydroxyapatite solubilization. Indeed, we have found that non-hydrolysable organic diphosphonates (P-C-P) that can competitively inhibit phosphatase-catalysed phosphate hydrolysis cause a marked reduction of osteoclastic bone resorption.

It is also clear from the results that acid phosphatase release, although essential, is on its own insufficient for bone resorption and the dramatic increase of enzyme

activity in response to increased cyclic AMP accumulation or G protein stimulation is not followed by the stimulation of bone resorption. This has taken us a step further in understanding the mechanism of bone resorption. Thus we believe that resorption results from a complex of interacting activities of the osteoclast. These include both motile and secretory processes; each activity is essential for bone resorption but insufficient in itself. The release and action of acid phosphatase is one such mode of osteoclast activity and its abolition can serve as a useful basis for active pharmacological intervention.

*Regulation of acid phosphatase secretion by ambient calcium: a negative feedback mechanism*

During the process of resorption, the osteoclast generates and is consequently exposed to high local concentrations of ionized calcium. The ambient calcium concentration in the resorptive hemivacuole can rise up to 20 mM (Malgaroli, Maldolesi, Zamboni-Zallone & Teti, 1989). We show here that the exposure of actively resorbing osteoclasts to increased extracellular calcium concentrations causes a marked reduction in the rate of enzyme release together with abolition of resorptive activity. We have demonstrated the elevation of extracellular calcium transduces a rapid rise of the intracellular free calcium concentration to account for the inhibitory effects on osteoclast function (Zaidi *et al.* 1989b; Datta *et al.* 1989). We have also shown that 'calcium-activated' intracellular calcium elevation is a voltage- and dihydropyridine-insensitive phenomenon (Zaidi *et al.* 1989b).

This study therefore provides a second example of the inhibition by extracellular calcium (and intracellular calcium) of secretion of macromolecules from single cells. The situation is similar to that first described for parathyroid hormone-secreting chief cells, whereby as a physiological response to impending hypercalcaemia, the cell regulates parathyroid hormone secretion by monitoring changes in plasma calcium (Shoback, Thatcher, Leombruno & Brown, 1984; Nemeth, Wallace & Scarpa, 1986). There are, however, fundamental differences between the two responses. While parathyroid chief cells respond to changes in plasma calcium in steps of 0.1 mM, osteoclasts are far less sensitive and can sense fluctuations only when the extracellular concentration rises above 4 mM. Furthermore, parathyroid chief cells have a divalent cation sensitive 'receptor' linked to a phospholipase C-inositol 1,4,5-trisphosphate intracellular calcium redistribution system; the stimulation of this pathway causes a shortlived calcium signal that inhibits hormone secretion. This does not appear to be the case with osteoclasts, as  $Mg^{2+}$  fails to mimic calcium effects on intracellular calcium, enzyme release and bone resorption. The mechanism underlying the effect of extracellular calcium on the osteoclast is unclear, but may be due to calcium influx via a special type of calcium channel that is activatable by high extracellular calcium (Zaidi *et al.* 1989b). Whether or not this hypothesis is substantiated, the effects described here are physiologically relevant. The most obvious implication is that there exists a self-regulatory mechanism for the feedback control of osteoclast function: excess resorption will elevate ambient calcium and this, by elevating free cytosolic calcium, will limit further secretion and resorption. This hypothesis is strengthened by our observations that enzyme release from actively resorbing osteoclasts is significantly lower than that from cells incubated on plastic substrate.

Osteoclasts on plastic are not resorbing bone, and thus release into the medium uncontrolled quantities of enzyme. On the other hand, resorbing osteoclasts secrete less enzyme because the high levels of calcium generated from bone as a result of resorption exert a negative feedback on enzyme release.

*Intracellular regulation of acid phosphatase secretion: role of G proteins, cyclic AMP and cytosolic calcium*

Cells secrete enzymes by exocytosis, a process that is dependent on the fusion of cell membranes with those of enzyme-containing granules and subsequent fission of the fused membrane (reviews: Baker & Knight, 1986; Mason & Sattelle, 1988). Exocytosis can either be continuous ('non-regulated') or triggered ('regulated') (Baker & Knight, 1986). Continuous exocytosis is not subject to specific control; triggered exocytosis may or may not be associated with an elevation of intracellular calcium (Rink, Sanchez & Hallam, 1983; Baker & Knight, 1986). This is particularly true in most nervous and endocrine tissues. In clear contrast, our results with extracellular calcium and ionomycin indicate that the elevation of cytosolic calcium in the osteoclast is associated with a reduced rate of basal (or continuous) enzyme secretion. Two major implications follow. Firstly, it is clear that elevated cytosolic calcium levels can in some instances inhibit exocytosis, another example being that of the parathyroid chief cell (also see above) (Nemeth *et al.* 1986). However, a negative influence of elevated cytosolic calcium on enzyme exocytosis should not be taken to imply that 'continuous' exocytosis in osteoclasts is calcium independent. Indeed low levels of ionized calcium will be required for the active basal exocytosis (Judah & Quinn, 1978). Secondly, in contrast to previous assumptions, we show that continuous exocytosis is also inhibitable. A somewhat similar situation had previously been observed in actively secreting liver cells where mitosis caused a complete cessation of both continuous and triggered exocytosis (Warren, 1985).

The exocytosis of enzymes is also controlled by guanine nucleotide binding proteins (G proteins) (Burgoyne, 1987) acting as key signal transducers (Neer & Clapham, 1988). We have found that the activation of a  $G_s$ -like protein by cholera toxin (Gilman, 1987) or aluminium fluoride ions (Sternweis & Gilman, 1982) or the elevation of cellular cyclic AMP levels by dibutyryl cyclic AMP or forskolin lead to a marked 100-fold elevation of acid phosphatase release. This has been taken to imply that a cholera toxin-sensitive  $G_s$ -like protein triggered enzyme release, possibly via adenylate cyclase stimulation. (This is an established pathway for signal transduction in the osteoclast (Abeyasekera, Datta, MacIntyre, Moonga, Patchell & Zaidi, 1989).) We believe that this form of 'triggered' exocytosis is G protein dependent and calcium independent. A similar situation is seen in mast cells, where it is suggested that an unknown G protein is involved in membrane fusion, and an increase of intracellular calcium is neither necessary nor sufficient for secretion (Neher, 1988).

When considered in relation to its effect on osteoclastic resorption, the lack of a significant effect of pertussis toxin on enzyme release appears surprising. We have previously demonstrated that a pertussis toxin-sensitive G protein is involved in osteoclast signalling, and that this links calcitonin receptor stimulation with the phospholipase C-inositol 1,4,5-trisphosphate-(diacylglycerol)-calcium redistribution system (Abeyasekera *et al.* 1989). Thus the elevation of intracellular calcium,

expected as a consequence of the activation of the putative pertussis toxin-sensitive G protein, could indeed lower the exocytotic rate. Nevertheless, over the 18 h incubation time diacylglycerol production is likely to uncouple the G protein (by activating protein kinase C) resulting in decreased calcium mobilization (Maruyama, 1989), and as a result, a reduction in the level of inhibition of enzyme release.

Finally, the best possible explanation for the negative correlation between bone resorption and enzyme release following G protein activation or cyclic AMP generation is as follows. Although enzyme release is essential for the resorptive process, it is insufficient in itself. The activation of G proteins or cyclic AMP generation leads to the inhibition of various modes of osteoclast activity that are essential for bone resorption, such as osteoclast motility (Zaidi, Datta, Moonga & MacIntyre, 1990). The net effect is the inhibition of resorption. However, the stimulation of one such mode of activity, for example, enzyme release, is in no way sufficient to reverse the inhibition of resorption produced by inhibition of one or more modes of osteoclast activity.

In conclusion, we have established that both resorbing and non-resorbing osteoclasts release acid phosphatase, a hydrolytic enzyme that appears to be essential for pyrophosphate removal, the necessary first step in bone resorption; that the release of acid phosphatase is regulated by the level of extracellular calcium generated locally as a consequence of resorptive activity; and that enzyme secretion, in general, is the result of a complex of interacting intracellular mechanisms which, in the case of the osteoclast, involves at least two G proteins, intracellular calcium and cyclic AMP.

M. Z. acknowledges the support of Research into Ageing, the Arthritis and Rheumatism Council and the Leverhulme Trust. The authors thank Professor T. M. Cox (Cambridge) for preparing and purifying the antiserum. EHDP was a gift from Norwich Eaton (USA).

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